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Cardioprotective mitochondrial binding by hexokinase I is induced by a hyperoxic acute thermal insult in the rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Due to the substantial photosynthetic biomass in their habitat, salmonids such as the rainbow trout (*Oncorhynchus mykiss*) can be subject to hyperoxia in addition to high temperatures associated with climate change. Both stressful conditions increase the incidence of damaging reactive oxygen species (ROS). The mitochondrial association of hexokinase has been shown to increase in the hearts of certain fish experiencing hypoxia in a putative cardioprotective response to oxidative stress. In this study, the mitochondrial association of hexokinase I (HKI) and markers of oxidative damage and metabolic stress were probed to elucidate the cardioprotective role of hexokinase in the rainbow trout. Results showed that the co-administration of hyperoxia and hyperthermia increased the ventricular mitochondrially-bound fraction of HKI, whereas exposure to hyperthermia in normoxia had no effect; in the combined condition there was little evidence of increased stress. A second *in vitro* study using ventricular strips and isolated cardiomyocytes was undertaken to reconcile the cardioprotective role of HK in the rainbow trout with findings in mammalian studies, confirming that mitochondrial association of HK maintains aerobic efficiency and inhibits apoptosis. Finally, protein sequence analysis suggested that the physiological contributions of HKI and HKII in salmonids vary from those in mammals, further explaining the dynamic nature of the traditionally-inert HKI. Together, these findings help to explain the broader functions of HKI in the salmonid heart, and illustrate the role of complex environmental conditions in defining physiological responses.

1. Introduction

The co-occurrence of environmental hyperoxia and high temperatures is well-recognized, particularly in tropical and sub-tropical freshwaters. In these areas with substantial aquatic photosynthetic biomass, waters with low flow can exceed 200% air saturation (DO_2) (35 °C in a tropical pond; (MacCormack et al., 2003), or even higher (c.a. 375% DO_2 at 28 °C in a subtropical pond) (Jiménez et al., 2003) depending on water depth and level of solar irradiation. Temperate waters such as the Miramichi system in New Brunswick, Canada, can already reach well over 30 °C in the summer months (Caissie et al., 2012). Although oxygen measurements in the Miramichi are not available, they are likely comparable to those of subtropical waters reaching 28 °C and c.a. 160% oxygen saturation daily in the summer (e.g., Xu and Xu, 2015). Climate change threatens to further raise water temperatures in temperate regions (Caissie et al., 2014), which may increase the system's biomass even more (Feuchtmayr et al., 2009), increasing the likelihood of combined hyperthermic and hyperoxic stress.

Cardiomyocytes (CMs) from fish, such as the rainbow trout (*Oncorhynchus mykiss*), have long been a topic of interest due to their unique physiology. They are relatively tolerant of hypoxia much like fetal or neonatal mammalian CMs (Patterson and Zhang, 2010) due to both a relatively low metabolic rate and high glycogen content. Salmonid CMs have a narrow and elongated morphology with well-organized sarcomeric structure (Karro et al., 2017), in contrast with the rounded shape and disorganized contractile machinery of young mammalian CMs. These cells are metabolically specialized, as piscine CMs are highly dependent on oxidative metabolism but their mitochondria are well-separated from the sarcolemma (Karro et al., 2017). Since they largely lack transverse tubules, there is a considerable cytosolic gap between extracellular nutrients and the mitochondria they must reach to maintain cellular metabolic activity. This gap is functionally analogous to the cytosolic gap in rat and human CMs, which is shorter due to the presence of transverse tubules but rendered considerably more tortuous by a denser, more extensive cytoskeleton and sarcoplasmic reticulum (Karro et al., 2017). The recycling of ADP to the mitochondria can also be subject to a bottleneck at the outer

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mitochondrial membrane specifically in fish CMs, but this is mitigated by the close coupling of the highly-conserved glycolytic enzyme hexokinase (HK) to mitochondrial respiration (Karro et al., 2017). Hexokinase binds to the outer mitochondrial membrane at the voltage-dependent anion channel (VDAC), driven at least partially via pro-survival signaling through the Akt pathway. Increased mitochondrial HK (mtHK) density is accomplished in part by increasing total hexokinase expression to passively increase the bound fraction (Vander Heiden et al., 2001). In addition, a higher relative fraction of HK can be induced to bind to the outer mitochondrial membrane through post-translational modifications. The more mobile hexokinase II (HKII) isoform is directly phosphorylated by Akt and therefore its translocation patterns have been extensively elucidated; however, hexokinase I (HKI) does not share the Akt target consensus sequence (see review by Roberts and Miyamoto, 2015). The precise mechanisms underlying the mitochondrial binding of HKI are still unclear (Regenold et al., 2012), however both isoforms are sensitive to allosteric inhibition and removal from the mitochondria by their product glucose-6-phosphate (Sui and Wilson, 1997).

Mitochondrial binding by HK prevents mortality by inhibiting pro-apoptotic factor (Bcl-2 family)-mediated opening of the mitochondrial permeability transition pore (mPTP) (Arora and Pedersen, 1988; Calmettes et al., 2016; Kodde et al., 2007; Pedersen et al., 2002; Rosano, 2011; Schindler and Foley, 2013; Sun et al., 2008; Zhuo et al., 2015). Furthermore, mtHK also increases metabolic efficiency by providing a local source of ADP for the mitochondria (Calmettes et al., 2016; Da-Silva et al., 2004; Meyer et al., 2006; Pastorino et al., 2005; Rosano, 2011; Schindler and Foley, 2013; Sun et al., 2008). Little characterization of the mPTP and its associated proteins has been undertaken in fish, although the available evidence suggests that the teleost mPTP is similar in structure and function to its mammalian homologue (reviewed by Vianello et al., 2012). This readily-available ADP decreases the proton motive force and prevents excess mitochondrial superoxide production (Banh et al., 2016). Superoxide, like other reactive oxygen species (ROS), induces oxidative stress, which has a wide range of potential physiological effects including cardiac arrhythmogenesis (Tse et al., 2016), which is a likely a major cause of high temperature mortality in fish (Brijs et al., 2015; Chen et al., 2015; Clark et al., 2008; Ekström et al., 2016; Farrell, 2002). The importance of mtHK is especially evident in rainbow trout cardiomyocytes given their reliance on HK to maintain ADP return to the mitochondria (Karro et al., 2017). Mitochondria in fish muscle also show significantly higher intrinsic rates of ROS leakage than those in mammalian tissue (Banh et al., 2016), further necessitating mtHK-mediated cardioprotection.

Both hyperoxic and hyperthermic exposures can increase production of ROS (Abele et al., 1998; Abele and Puntarulo, 2004; Buchner et al., 2001). Consistent with a cardioprotective role of mtHK against oxidative stress, a higher cardiac mtHK fraction was implicated in a rapid response to *in vitro* hypoxia in the heart of an Amazonian armored catfish (Treberg et al., 2007). Several recent studies have investigated the effect of environmental hyperoxia compounded with acute hyperthermia on various physiological and cardiac performance metrics (Brijs et al., 2015; Devor et al., 2015; Ekström et al., 2016). Based on the functions of HK described above, it is possible that the compounded induction of oxidative stress *via* hyperoxia and hyperthermia in the salmonid heart could be at least partially countered by increased mtHK density. Furthermore, as physiological responses governing cardiorespiratory function could be affected by the presence of ROS before oxidative damage is evident, reversible HK localization could provide a sensitive indication of disrupted cellular homeostasis.

In this study, we aimed to characterize mitochondrial hexokinase I (mtHKI) binding in the ventricles of rainbow trout (*Oncorhynchus mykiss*) exposed to both aerated and hyperoxic thermal episodes, with the hypothesis that mtHKI binding would increase under a hyperoxic high-temperature event and ameliorate oxidative stress relative to a normally-aerated thermal ramp. We additionally performed *in vitro* experiments examining the effect of mtHK binding on ventricular muscle function and cardiomyocyte respiration and mortality, by comparing the effect of lonidamine (LND)-mediated blocking of mtHKI association with the ionophore-mediated mitochondrial uncoupling of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP). We hypothesized that LND-mediated mtHKI dissociation would increase CM oxygen consumption due to mitochondrial uncoupling, as the accumulated proton gradient escapes through the open mPTP. Furthermore, we expected that the LND-affected rate of oxygen consumption would mirror that of FCCP treatment, as the proton gradient would be dissipated similarly in both treatments albeit through different mechanisms. Finally, we hypothesized that dissociation of mtHKI using LND would prove more lethal to cardiomyocytes than FCCP treatment due to the induction of apoptotic pathways from an unprotected mPTP. Our results suggest that ventricular mtHK binding is not a factor in the cardiac response to acute thermal exposures alone, but that rainbow trout increase the ventricular mtHKI fraction when an acute thermal challenge is compounded with hyperoxia. This finding, when combined with a transient decrease in plasma lactate levels and an increase in ventricular ubiquitinylated conjugates, suggests a synergy in responses, or else that a certain environmental stimulus threshold is required to mount a cardioprotective response. This regulation may represent part of a response to counter incipient oxidative stress, although further work is necessary to fully characterize the physiological implications of this exposure.

2. Materials and methods

2.1. Animals

Female two year old rainbow trout were held at Mount Allison University (Sackville, NB) in one 750 L tank of freshwater (total system volume of 2400 L at 16 ± 0.5 °C, recirculated at 18 L min^{-1} $tank^{-1}$ through a biofilter and UV light) and fed *ad libitum* with sinking pellets. All procedures were performed with the approval of the Mount Allison University Animal Care Committee. Fish were not fed for 48 h prior to experimentation.

All temperature treatments were carried out in 3 × 300 L recirculating tanks. Three animals at a time (N = 6 each treatment) were introduced to a tank at 16 ± 0.5 °C 24 h before the trial began. The study consisted of three treatments. The control (hyperthermic(−), hyperoxic(−)) treatment where temperature was held at 16 °C for 13 h, with the tank continuously aerated to a dissolved oxygen partial pressure (P_{O2}) of 149 ± 2 mmHg (c.a. 95% air saturation). The second treatment (hyperthermic(+), hyperoxic(−)) consisted of an aerated temperature ramp where water temperature was raised from 16 to 27 °C linearly over 12 h, and then held for 1 h at 27 °C while keeping P_{O2} constant at 149 ± 2 mmHg during the ramp. Finally, fish were exposed to a hyperoxygenated ramp (hyperthermic(+), hyperoxic(+)) which was identical to the aerated ramp treatment except that P_{O2} was held at 315 ± 3 mmHg (c.a. 205% air saturation at 27 °C) by bubbling with 100% O_2.

At the end of each treatment or for *in vitro* experiments (below), fish were placed in an anaesthetic bath (300 mg L^{-1} tricaine methanesulfonate buffered with 600 mg L^{-1} $NaHCO_3$) until ventilation ceased. Blood samples were collected using a 23-gauge needle and 1 mL syringe treated with Cortland saline containing (in mmol L^{-1}): NaCl (143). $CaCl_2$ (0.88), $MgSO_4$ (0.90), KCl (3.35), NaH_2PO_4 (2.25), $NaHCO_3$ (5.50), HEPES (0.010), glucose (2.78), sodium pyruvate (4.00), and 100 U mL^{-1} sodium heparin, pH 7.5. Fish were sacrificed by cervical transection. Hearts were quickly dissected and placed in an ice-cold bath of incubation buffer containing (in mmol L^{-1}) NaCl (100), KCl (10.0), KH_2PO_4 (1.2), $MgSO_4$ (4.0), $CaCl_2$ (2.0), HEPES (10.0), pH 6.9.The bulbus arteriosus and atrium were separated from the ventricle and residual blood removed. Blood samples were centrifuged at 4 °C for 10 min at 4000 × *g*. Plasma was removed, diluted 1:9 (v:v) in

6% perchloric acid, centrifuged at 4 °C for 10 min at 7000 × *g*, and the supernatant assayed for lactate (MacCormack and Driedzic, 2007).

2.2. Mitochondrial isolation

Ventricular mitochondria were isolated according to established protocols (Schindler and Foley, 2013; Treberg et al., 2007). Fresh ventricles from treated fish were finely chopped and lysed 1:4 (w:v) in mitochondrial fractionation buffer containing (in mmol L^{-1}) sucrose (250), HEPES (10.0), EGTA (1.0), pH 8.0, in a test tube using a Teflon pestle attached to a handheld drill in 3 × 10 s bursts. This homogenate was centrifuged at 4 °C for 10 min at 700 *g* to pellet insoluble debris. An aliquot of supernatant was assayed undiluted for malondialdehyde (MDA) levels using a Bioxytech® MDA kit (#20144, Oxis International Inc., Foster City, CA). The remaining supernatant was centrifuged at 4 °C for 15 min at 7000 *g* to pellet mitochondria, which were washed with 2 × 500 μL portions of ice-cold mitochondrial fractionation buffer and resuspended in 50 μL of the same. Protein content was determined using a BCA assay kit (Thermo-Fisher Scientific, Rockford, IL, USA). SDS-PAGE samples of each extract were prepared at a final protein concentration of 1 mg mL^{-1} using mitochondrial fractionation buffer, a few crystals of phenylmethanesulfonyl fluoride (PMSF), and 2X SDS-PAGE loading buffer (containing 125 mmol L^{-1} Tris-HCl pH 7.5, 20% (w/v) glycerol, 0.1% (w/v) bromophenol blue, 4% (w/v) SDS, and 500 mmol L^{-1} dithiothreitol, pH 7.5). Samples were vortexed, boiled for at 95 °C for 10 min, and then cooled and stored at − 80 °C.

2.3. Protein detection

To probe for mtHKI density, samples were loaded to deliver 15 μg per well protein in 17-well Bolt® 4–12% Bis-Tris Plus Gels (Thermo-Fisher Scientific, Waltham, MA, USA) with one lane containing a biotinylated protein ladder (#7075, Cell Signaling Technology, Danvers, MA, USA). Electrophoresis was carried out using a Mini Gel Tank (Thermo-Fisher Scientific) and wet transfer was performed using the Thermo-Fisher Mini Blot Module with Bolt® supplies according to manufacturer instructions, and blocking according to Cell Signaling Technology. Membranes were probed using rabbit monoclonal antibodies for hexokinase I (#2024, batch #3, Cell Signaling Technology) and VDAC (#4661, batch #6, Cell Signaling Technology). Detection of HKII was also attempted using a rabbit monoclonal antibody (#2867, batch#, Cell Signaling Technology), but no bands were detected owing to low antibody specificity for the *O. mykiss* HKII sequence. Blotting was carried out according to manufacturer directions using 1:1000 primary antibody, 1:3000 secondary anti-rabbit IgG, HRP-linked antibody (#7074, batch #25, Cell Signaling Technology), and 1:3000 anti-biotin, HRP-linked antibody (#7075, batch #30, Cell Signaling Technology) to detect the biotinylated ladder. Blots were imaged using SignalFire™ Plus ECL Reagent (Cell Signaling Technology) in a Molecular Imager® VersaDoc™4000 MP (Bio-Rad Laboratories, Hercules, CA, USA) with ImageLab™ software. Hexokinase I bands were quantified relative to both their respective VDAC band and to the control treatment using ImageJ (National Institute of Mental Health, Bethesda, MD, USA). Significance was determined by one-way ANOVA/Tukey's HSD using SPSS 21 (IBM Corporation, Armonk, NY).

Dot blots were used to assess ubiquitin levels in unfractionated ventricle homogenate supernatants. Samples containing 0.40 μg protein were directly loaded on a nitrocellulose membrane and dried 1 h. The membrane was blocked in 5% (w/v) BSA in TBS and then blotted using 1:2500 anti-ubiquitin IgM (BML-PW8805, batch # 09051237, Enzo Life Sciences, Farmingdale, NY, USA) and 1:20 000 goat anti-mouse IgM, HRP-linked antibody (ab97265, batch #GR144922-5, Abcam Inc., Toronto, ON). Membranes were treated with ECL select reagent (RPN2235V1/2, batch #9630990, GE Healthcare UK Ltd., Buckinghamshire, UK) and imaged as previously described. Each signal was quantified relative to a pooled pseudostandard curve and analyzed by one-way ANOVA/Tukey's HSD using SPSS 21.

2.4. Cardiac contractility

As we noted an increase in ventricular mtHK in the combined hyperthermia/hyperoxia condition, we sought to examine its physiological significance using *in vitro* pharmaceutical dissociation of the complex. However, there is no universally-accepted small molecule to separate HK from the mitochondria. Lonidamine has been long known to decrease the mtHK bound fraction (Oudard et al., 1995), albeit with several notable off-target effects including inhibition of Complex II resulting in increased ROS production (Guo et al., 2016). Little kinetic information about LND-mediated dissociation of mtHK has been gathered. Given available inhibitory data across taxa, consistent from protozoans to mammals, *c.a.* 15–20% inhibition of maximal HK activity can be expected due to the 150 μM dose (Chambers et al., 2008; Floridi et al., 1981). However, disruption of the membrane potential and thus oxidative phosphorylation occurs well below this concentration (Belzacq et al., 2001), further supporting LND's purported mechanism of HK dissociation.

Two ventricular muscle strips measuring c.a. 0.5 mm wide by 8 mm long were cut sagittally from the posterior wall of untreated hearts. Each strip was clamped on one end and tied with a silk suture at the other to a 0.5 g isometric force transducer (Harvard Apparatus, South Natick, MA, USA) in a 20 mL bath of incubation buffer with 2.0 mmol L^{-1} $CaCl_2$ and 5 mmol L^{-1} glucose at 16 °C. Transducer outputs were connected to a PowerLab 8/35 data acquisition system (ADInstruments Inc., Colorado Springs, CO, USA), and data were recorded with LabChart 8 Software (ADInstruments Inc.). The setup allowed for paired experiments treating strips (N = 5 each treatment) from the same individual with either 150 μmol L^{-1} lonidamine in 0.30% (v/v) DMSO, or a 0.30% DMSO sham. After stretching for optimal peak contractile tension development (F_{max}), treated strips equilibrated for 30 min under continuous monophasic electrical stimulation at 60 V, 10 ms duration at 0.4 Hz. Preparations were subjected to a force-frequency challenge, whereupon stimulation increased in 0.2 Hz increments every 30 s, terminating at 2.0 Hz. Treatments were compared by multiple linear regression analysis using SPSS 21.

2.5. Isolated cardiomyocyte respirometry

Ventricular cardiomyocytes from untreated fish were isolated using an established protocol (Legate et al., 1998), with slight modifications. Briefly, chopped ventricles were rinsed for 15 min with constant stirring in 4 °C Ca^{2+}-free isolation solution containing (in $mmol^{-1}$) NaCl (100), KCl (10.0), KH_2PO_4 (1.2), $MgSO_4$ (4.0), and HEPES (10.0), pH 6.9, with additional 30 mmol L^{-1} taurine and 20 mmol L^{-1} glucose. Media was then refreshed with additional 0.75 mg mL^{-1} collagenase type 1A, 0.5 mg mL^{-1} trypsin type IX.S, and 0.75 mg mL^{-1}BSA, and allowed 1 h to digest with further mincing at 30 min. After incubation, the preparation was gently triturated to produce a single-cell suspension and passed through a 41 μm nylon net filter and the filter rinsed with incubation medium containing (in mmol L^{-1}) NaCl (155), KCl (5.0), NaH_2PO_4 (1.0), $MgSO_4$ (2.0), and HEPES (10.0), pH 7.6. Filtrate was centrifuged at 4 °C for 5 min at 1500 × *g*. The supernatant was discarded by pipette, and the pellet resuspended in 250 μL incubation medium containing 125 μmol L^{-1} $CaCl_2$ for 5 min, with successive additions of 250 μL medium containing 0.25, 0.5, 1, and 2 mmol L^{-1} $CaCl_2$. Cells were then centrifuged at 4 °C for 5 min at 1500 × *g* and resuspended in incubation media containing 2 mmol L^{-1} $CaCl_2$ to a final concentration of 50 mg cells mL^{-1}. Cell viability was measured using with equal parts cell suspension and 0.4% (w/v) Trypan Blue, 0.81% NaCl, and 0.06% K_2HPO_4. Cardiomyocyte preparations exceeding 15% initial mortality were not used for respirometry experiments. The cell suspension was then divided between two OX1LP respirometry chambers (Qubit Systems Inc., Kingston, ON)

outfitted with dissolved oxygen electrodes and set to stir continuously.

Preparations were either treated with a 0.30% (*v*/v) DMSO sham or 150 μmol L^{-1} lonidamine in 0.30% DMSO (N = 6 each treatment). Oxygen consumption ($\dot{M}O_2$) was then measured for 25 min and cell viability was reassessed post-treatment. Net cardiomyocyte viability and maximum linear $\dot{M}O_2$ were compared between treatments in the sham/lonidamine experiment by paired one-tailed Student's *t*-test using Microsoft Excel 2007. To separate mortality associated with mitochondrial uncoupling from that with mPTP opening, a second experiment compared $\dot{M}O_2$ and viability in suspensions exposed to either 150 μmol L^{-1} lonidamine, 40 μmol L^{-1} of the mitochondrial uncoupler carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) (Maro et al., 1982), or both 150 μmol L^{-1} lonidamine and 40 μmol L^{-1} FCCP together, all in 0.30% DMSO (N = 6 each treatment). As only two of three treatments could be measured in parallel due to equipment limitations, a nested ANOVA was first used to eliminate individuals as a factor. Treatments were then grouped together and analyzed by one-way ANOVA/Tukey's HSD using SPSS 21 to find significance between treatments.

2.6. Protein sequence analysis

Significant differences in mtHKI density were noted across treatments (see below). To qualitatively account for possible difference between human or mammalian models and salmonids in the regulation of HKI and HKII, the sequences of human HKI (accession no. AAA52646.1) (Nishi et al., 1988) and HKII (AAH64369.1) (Strausberg et al., 2002) were compared with predicted sequences of HKI (XP_014035682.1) and HKII (XP_014026417.1) (Davidson et al., 2010) of Atlantic salmon (*Salmo salar*), a close relative of rainbow trout. All sequences were obtained from the NCBI online database. Alignments by Multiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar, 2004) were carried out using default settings to estimate residue identity, positive homology, and gaps in the sequence. No statistics were performed on alignment results.

3. Results

We examined the cardiac mitochondrial density of HKI and markers of oxidative and metabolic stress in trout exposed to an aerated control exposure, an aerated (normoxic) thermal insult, or a hyperoxic thermal insult. Should mtHK provide a cardioprotective effect in the face of combined hyperoxia and thermal stress, a higher relative mitochondrial fraction of HK would be expected. Indeed, immunoblotting of the mitochondrial fraction revealed a significant difference in mtHKI binding ($p = 0.037$, Fig. 1A), with higher mtHKI levels in the hyperoxic than both the aerated heat exposure ($p = 0.021$) and the control ($p = 0.029$), but there was no difference in ventricular MDA levels ($p = 0.176$, Fig. 1B). Higher levels of ubiquitinylated conjugates were observed in the hyperoxic exposure than either the aerated exposure ($p = 0.041$, Fig. 2A) or control ($p = 0.0021$), while there was no difference between the latter two ($p = 0.318$), strongly suggesting changes to protein turnover. There was a significant effect of treatment on plasma lactate levels ($p = 0.032$, Fig. 2B) with the aerated exposure tending higher than either hyperoxic ($p = 0.054$) or control ($p = 0.053$) treatments, suggesting a minor reliance on anaerobism.

To confirm the cardioprotective role of mtHK, we carried out *in vitro* experiments using a LND concentration which dissociates HK from the mitochondria without inhibiting HK activity (Chambers et al., 2008). If mtHK is essential for maintained CM health due to prevention of ROS production and blockage of mPTP, then LND-mediated dissociation of mtHK with minimal inhibitory impact on enzymatic activity would be expected to have little effect on muscular force production. Furthermore, the LND-opened mPTP would be expected to decrease oxidative metabolic efficiency due to dissipation of the accumulated H^+ gradient. Finally, the administration of LND would be expected to decrease cell viability, ostensibly through oxidative damage and mPTP-related apoptotic induction. Ventricle strip preparations did not differ in peak force production ($p = 0.54$, Fig. 3A), resting tension ($p = 0.079$, Fig. 3B), or in contractile kinetics (time to peak tension and time to half-relaxation; not shown). Incubation of isolated cardiomyocytes with LND increased their $\dot{M}O_2$ by 60% ($p = 0.00015$, Fig. 3C) and mortality by 330% ($p = 0.00037$, Fig. 3D). We expected that exposure to the ionophore FCCP would have a lower associated rate of mortality as it would not allow for an unguarded mPTP (i.e. FCCP-increased mortality would be due to metabolic insufficiency alone). Separate or co-administration of FCCP would be expected to have a similar but non-cumulative effect on respiration (as a complete dissipation of the proton motive force cannot be exacerbated). The application of FCCP did not change $\dot{M}O_2$ on its own or in combination with LND ($p = 0.67$, Fig. 3E), but induced cumulative mortality when compounded with LND exposure ($p = 0.0072$, Fig. 3F), demonstrating identical mitochondrial uncoupling effects between LND and FCCP through different mechanisms.

Alignment of the whole sequences of Atlantic salmon and human HKI and HKII revealed expected levels of variation between isoforms and species; the same isoform across species shared higher positive homology than different isoforms within the same species (Suppl. Table 1). However, one of the regions of the lowest homology and conserved residue identity is found in the regulatory subunit (N-terminal half) centered around human HKII D341, with no other sequence sharing even a homologous residue (Fig. 4). Furthermore, the human HKI N345 residue, important in the regulation of VDAC (Rosano, 2011), had homologs in salmonid HKI and HKII, but no analogous residues in

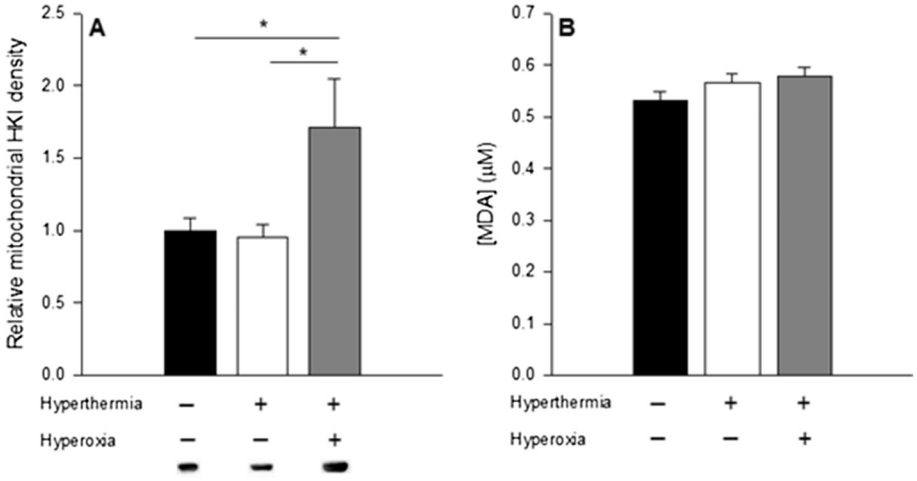

Fig. 1. Hyperoxic but not aerated acute thermal exposures induce increased mitochondrial binding by hexokinase 1 (A), but neither treatment induced significant oxidative damage relative to the control as measured by malondialdehyde (MDA) levels (B) (N = 6). Representative immunoblot bands for each column are displayed in (A). Values are plotted as mean ± SEM, significance indicated as $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

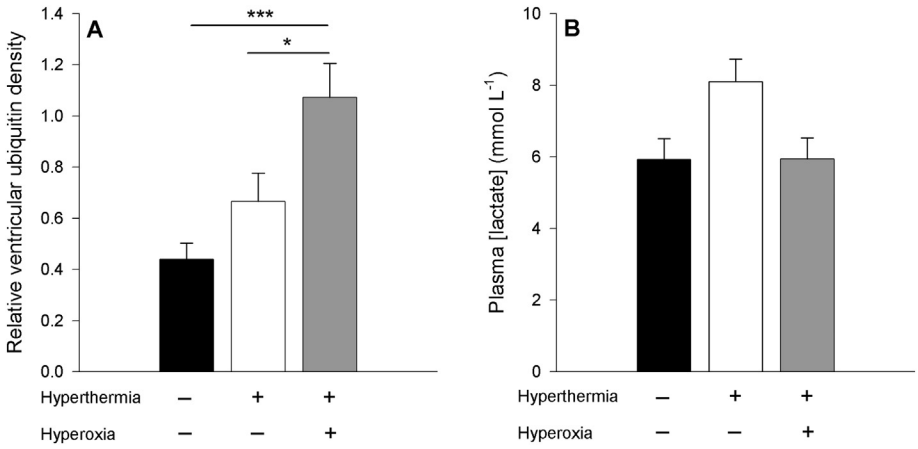

Fig. 2. Hyperoxic acute thermal exposure induces increased ventricular mitochondrial levels of ubiquitinylated conjugates relative to either the aerated thermal exposure or the control treatment (A), but plasma lactate levels were not significantly elevated in any treatment (B) (N = 6). Values are plotted as mean ± SEM, significance indicated as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

human HKII. These patterns of homology in this key area suggest a discrepancy between the domain's functionality between human and salmonid HKII similarly to that between human HKI and HKII.

4. Discussion

A hyperoxic acute heat exposure allows both higher maximum $\dot{M}O_2$ and aerobic scope in fish (Brijs et al., 2015), potentially allowing for greater ROS production at the mitochondria. Increasing mtHK activity, as seen in the hyperoxic thermal exposure, may facilitate the maintenance of low ATP/ADP ratios in the mitochondria (Treberg et al., 2007) and thereby prevent excessive mitochondrial ROS production by promoting electron transport chain efficiency (Arora and Pedersen, 1988; Calmettes et al., 2016; Da-Silva et al., 2004; Sun et al., 2008). A similar phenomenon is observed in the heart of armored catfish exposed to hypoxia, where the mitochondrial HK fraction increases in density (Treberg et al., 2007). This may represent a rapid and sensitive cardioprotective response in the teleost heart before any oxidative damage accumulates, as evidenced by the lack of difference between treatments in ventricular MDA levels. The increase in ventricular ubiquitinylated conjugates provides further evidence of disrupted homeostasis. Ubiquitinylation, as a correlative marker of protein damage, supports the evidence of hyperoxia inducing an additional cellular stress at high temperatures.

The observed tendency for higher plasma lactate levels in the aerated thermal exposure suggests increased reliance on anaerobic metabolism to maintain function. This finding is supported by a recent study that observed an increased $\dot{M}O_2$ in European perch (*Perca fluviatilis*) exposed to a hyperoxic rather than normoxic thermal ramp (Brijs et al., 2015), suggesting that some species can benefit from supplemental oxygen in an environmentally-relevant acute thermal episode. Additionally, the maintenance of plasma lactate in the hyperoxic treatment, coupled with the lack of change in ventricular MDA levels, suggests that trout were largely able to avoid both harmful oxidative damage and anaerobism for the duration of the experiment.

Hyperoxia is known to increase ROS production, which is implicated in a wide range of physiological effects, including arrythmogenesis (Tse et al., 2016). Our results suggest there is background signaling during an acute hyperoxic thermal exposure coordinating protein localization, expression, and turnover, even before oxidative damage has accumulated. Specifically, increased mtHKI density during combined hyperoxia and hyperthermia should limit both oxidative stress and apoptosis to protect cardiac muscle. These processes would then lower the risk of arrhythmogenesis and the associated disruptions in oxygen uptake and delivery that limit survival under physiological stress. Furthermore, the gradation in the increase of cardiac protein ubiquitinylation between the aerated and hyperoxic exposures suggests that the degree of response induced can be fine-tuned based on the signal; the amount of ROS produced would likely be compounded by both the hyperthermic and hyperoxic aspects of the exposure and could have induced redox-sensitive signaling in the CM. Our finding that mtHKI density only increases during a hyperthermic event when hyperoxia is also present suggests that either a certain threshold of ROS is needed to trigger this cardioprotective effect, or else that separate hyperoxic and hyperthermic signaling may synergize to increase mtHKI density. We were unable to detect a change in ROS between treatments using malondialdehyde as a proxy for oxidative damage, but this should not be taken to suggest that there is no difference in ROS production between treatments. The contributions of high temperature (Banh et al., 2016) and hyperoxia (Lushchak and Bagnyukova, 2006) to ROS production in fish are well-recognized. In this case, an increase in ROS production may have been masked by adequate antioxidant reserves such as glutathione or thioredoxins over the short time course of the experiment. Sustained or repeated insults may have resulted in higher levels of MDA or other markers of oxidative stress. Together, these results support previous findings of improved performance in terms of higher CTmax (Brijs et al., 2015; Ekström et al., 2016) or decreased plasma lactate (Devor et al., 2015) when hyperthermic events are compounded with hyperoxia.

As mtHKI levels seemed highly dynamic when considering the dogma of non-actively regulated mammalian HKI, an *in vitro* experiment was undertaken to confirm the cardioprotective nature of mtHK. We hypothesized that LND-mediated dissociation of mtHK would increase mortality in isolated CMs by leaving the mPTP open, allowing dissipation of the mitochondrial membrane potential as well as cytochrome C release to the cytosol, which initiates apoptotic processes. Moreover, we hypothesized that LND-mediated dissipation of the proton motive force would effectively short-circuit the electron transport chain and result in increased $\dot{M}O_2$ as metabolism continues at full capacity with little benefit in the form of ATP production. As the ionophore FCCP allows for dissipation of the mitochondrial membrane potential without opening of the mPTP (Pulselli et al., 1996), we finally hypothesized that the administration of FCCP would have a similar and noncumulative effect on $\dot{M}O_2$ as LND, but that mortality would be cumulative between the two treatments. Force production in the ventricular strip preparations was not affected by LND treatment, suggesting that inhibition of HK was not sufficient to depress metabolic flux enough to affect contractility, a highly costly activity in terms of energy consumption. Furthermore, the increase in oxygen utilization upon administration of LND or FCCP supported our hypothesis that metabolism was inhibited at the stage of oxidative phosphorylation, as substantial inhibition of hexokinase would prevent downstream use of O_2 to catabolize glycolytic end products. Our results closely matched our predictions, supporting the cardioprotective role of mtHK in salmonids. It is important to note that this experiment was not specific to HKI and may also have influenced HKII localization. The dynamic

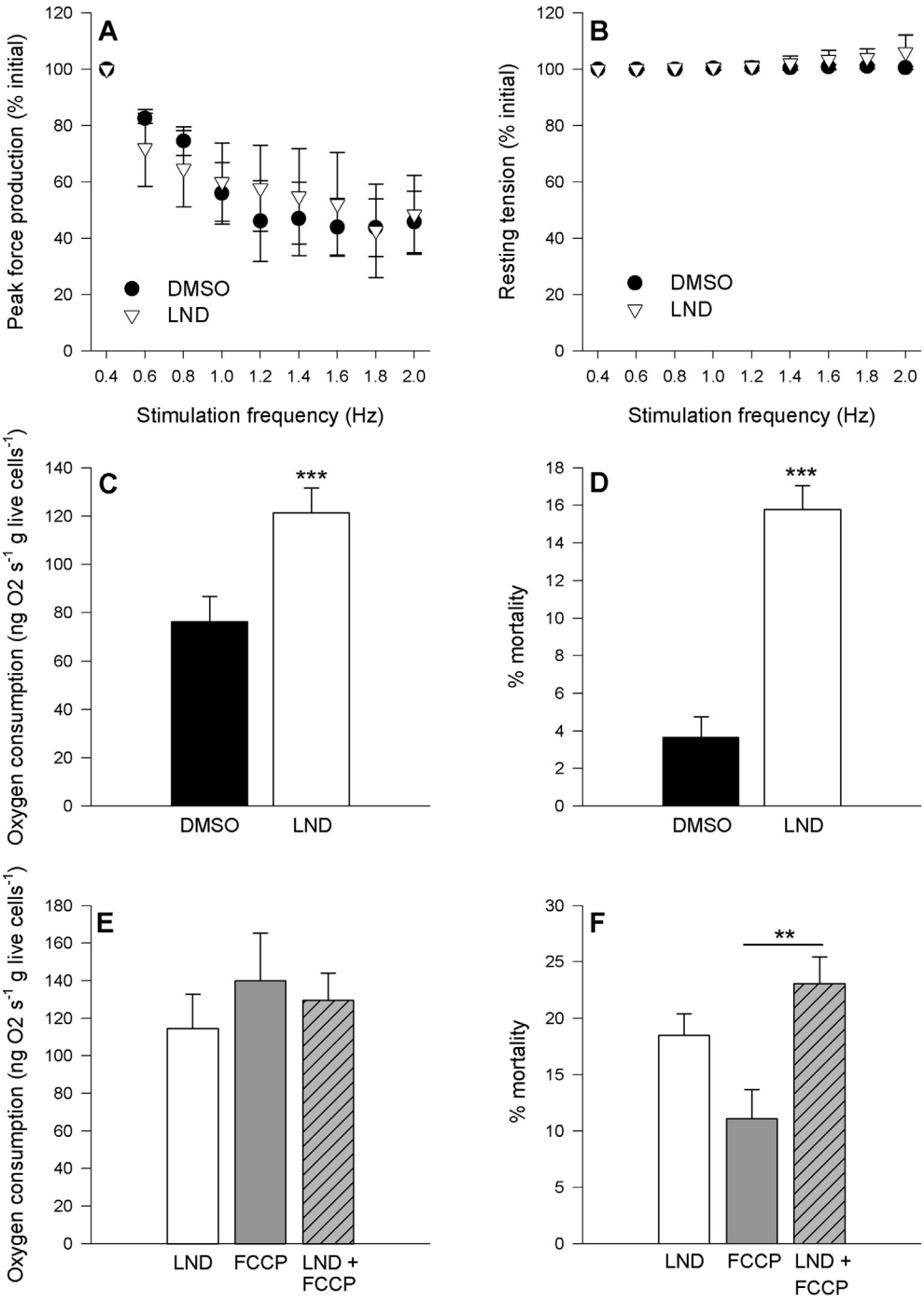

Fig. 3. *In vitro* experimental results of pharmacological mtHK dissociation in ventricular tissue. LND does not affect peak isolated ventricle strip isometric force production (A) or resting tension (B) (N = 5) of under increasing electrical stimulation frequency. Administration of lonidamine (LND) increases oxygen consumption rate (C) and mortality (D) in isolated cardiomyocytes (N = 6). LND and FCCP affect the rate of isolated cardiomyocyte oxygen consumption similarly when administered (E), but cumulatively increase mortality (F) (N = 6). Values are plotted as mean ± SEM; significance is indicated as $*p \geq 0.05$, $**p \geq 0.01$, $***p \geq 0.005$.

nature of salmonid ventricular HKI localization strongly suggests some degree of active regulation, specifically in a situation where cardioprotective measures are expected to be undertaken.

Sequences of HKI and HKII from humans were compared to those of the Atlantic salmon to better understand the observation of HKI movement to the mitochondrial fraction in this study, as HKI in mammals is commonly assumed to be mostly passively regulated by product inhibition, with little evidence of signaling-mediated translocation or allosteric control of activity (Roberts and Miyamoto, 2015). Similar levels of homology and identity of the same isoforms between species, and separately between isoforms of the same species was not unexpected. However, one of the areas of lowest homology was centered around D341 in human HKII. This residue, which is the centre of the target of the HKII antibody that was unsuccessfully used in this study to

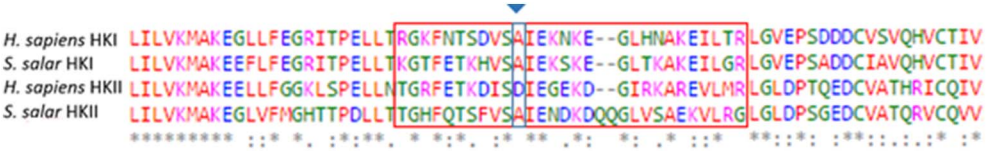
H. sapiens HKI LILVKMAKEGLLFEGRITPELLTRGKFNTSDVSAIEKNKE--GLHNAKEILTRLGVEPSDDDCVSVQHVCTIV
S. salar HKI LILVKMAKEEFLFEGRITPELLTKGTFETKHVSAIEKSKE--GLTKAKEILGRLGVEPSADDCIAVQHVCTIV
H. sapiens HKII LILVKMAKEELLFGGKLSPELLNTGRFETKDISDIEGEKD--GIRKAREVLMRLGLDPTQEDCVATHRICQIV
S. salar HKII LILVKMAKEGLVFMGHTTPDLLTTGHFQTSFVSAIENDKDQQGLVSAEKVLRGLGLDPSGEDCVATQRVCQVV

Fig. 4. MUSCLE alignment of HKI and HKII from *Homo sapiens* and *Salmo salar*. The blue box and arrow indicate the D341 residue commonly targeted by mammalian HKII-specific antibodies, while the red box indicates a surrounding area of notably low positive homology between sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

target *O. mykiss* HKII was not present in any of the other protein sequences studied. This region forms part of the regulatory subunit of HKII, which unlike other HK isoforms, also possesses catalytic activity (Roberts and Miyamoto, 2015). Furthermore, the amidyl functional group of the N345 residue in human HKI represents an important region of association with VDAC (Rosano, 2011); this residue has homologs in salmonid HKI and HKII (Fig. 4) but is replaced by a glycine residue lacking an analogous functional group in human HKII. Mitochondrial hexokinase binding is regulated in part by glycogen synthase kinase- and protein kinase C-mediated phosphorylation of VDAC which directly induces changes in tertiary structure (Pastorino et al., 2005; Sun et al., 2008). These results suggest that regulation of salmonid HKII may differ from that of the mammalian isoform due to key differences in its primary sequence. Therefore, fish may be more reliant than mammals on mtHKI for dynamic protection at the mPTP. These findings are supported by both the enhanced cardioprotective duty of HK in fish (Karro et al., 2017), and the increased relative rate of ROS leakage from mitochondria from fish when compared to mammals (Banh et al., 2016).

Further investigation into both mtHKI density as a function of differing ROS flux, and metabolic signaling during hyperoxic and hyperthermic exposures may help to elucidate the mechanisms underlying these curious physiological changes.

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Competing interests

The authors declare no competing interests.

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Author contributions

NIC and TJM planned experiments. NIC and KJW performed experiments. NIC analyzed data. NIC and TJM interpreted results and wrote the manuscript. All authors approved the final manuscript.

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